American Journal of
CLINICAL PATHOLOGY

TECHNICAL SUPPLEMENT

VOL. 2 JANUARY, 1938 No. 1

EDITOR-IN-CHIEF

R. A. KILDUFFE, Atlantic City Hospital, Atlantic City, New Jersey

EDITOR OF TECHNICAL SUPPLEMENT

W. S. THOMAS, M.D., Clifton Springs Sanitarium, Clifton Springs, New York

ASSOCIATES

M. BODANSKY, M.D., John Sealy Hospital and Department of Pathological Chemistry, University of Texas, Galveston, Texas.

I. DAVIDSOHN, M.D., Mt. Sinai Hospital, Chicago, Ill.

KANO IKEDA, M.D., Chas. T. Miller Hospital, St. Paul, Minn.

Contents

ORIGINAL ARTICLES

The Use of Bromthymol Blue and Phenol Red Mannitol Agar for the Isolation of Pathogenic Types of Staphylococci. G. H. CHAPMAN, C. W. LEIB AND L. G. CURIO 3

Determination of Blood Creatinine, etc. J. E. ANDES 12

Molasses Agar for the Isolation and Cultivation of Yeast Molds. SOL HABERMAN 20

The Determination of Blood Plasma and Spinal Fluid Proteins. G. W. JOHNSTON AND R. B. GIBSON 22

A New Contrast Mounting Medium in Different Colors. GUSTAV WILLENS 32

PUBLISHED PROCEDURES RECOMMENDED FOR TRIAL

An Adaptation of Van Slyke's Simplified Determination of Blood Urea Clearance 33

A Simple Method for Silver Impregnation of Reticulin 35

Stain for Myelin Sheaths Embedded in Paraffin 36

A Simple Method for Obtaining Anerobiasis 36

Individual Culture Dish with Increased Carbon Dioxide Tension. 37

Annotations, Minor Contributions, Queries 38

PUBLISHED BI-MONTHLY BY THE WILLIAMS & WILKINS COMPANY

MOUNT ROYAL AND GUILFORD AVES., BALTIMORE, U. S. A.

Made in United States of America

SUBSCRIPTION PRICE $1.50 PER VOLUME

All rights reserved

THE USE OF BROMTHYMOL BLUE AGAR AND PHENOL RED MANNITOL AGAR FOR THE ISOLATION OF PATHOGENIC TYPES OF STAPHYLOCOCCI*

GEORGE H. CHAPMAN, CLARENCE W. LIEB AND LILLIAN G. CURCIO

Clinical Research Laboratory, New York, N. Y.

It was shown[1] that, on alkaline proteose lactose agar containing 0.017 per cent bromthymol blue, 98.5 per cent of pathogenic staphylococci grow luxuriantly in 48 hours while 94 per cent of non-pathogenic staphylococci grow poorly or not at all. The medium was proposed for the isolation of probable pathogenic staphylococci from mixed cultures.

The basic principles of the medium were: (a) selective inhibition of non-pathogenic staphylococci by bromthymol blue; and (b) enhancement of growth of pathogenic staphylococci by lactose which is fermented by pathogenic and a few non-pathogenic strains. While certain facts suggested that pathogenic strains were also inhibited by this medium, the parallel results obtained by selecting and testing various types of colonies from rabbit blood agar plates seemed to disprove this idea. However, the impression persisted and an alternate isolation medium was sought. Carbohydrate fermentation media were considered.

Numerous workers have studied the relation between fermentation of carbohydrates and other properties of staphylococci. The conclusion to be drawn from most of the reports is that certain carbohydrates are fermented by most pathogenic staphylococci but that this property is also shared by some non-pathogenic strains. Recent workers have centered their attention on mannitol. Fermentation of this alcohol was found to be parallel with in vitro and pathogenic properties of staphylococci[2] and mannitol agar was selected for further study.

* Aided by grants from the Ophthalmological Foundation, Inc.

TABLE 1

RECOVERY OF PATHOGENIC TYPE STAPHYLOCOCCI FROM BROMTHYMOL BLUE AGAR AND PHENOL RED MANNITOL AGAR

PATIENT	SOURCE	BROMTHYMOL BLUE AGAR							PHENOL RED MANNITOL AGAR						
		Number	P	H	C	V	B	M*	Number	P	H	C	V	B	M
Cultures which gave *similar* results with both media: 65 (58.5%)															
Mar	Nose	Many							Many						
Whi	Nose	Many							Many						
Noo	Nose	Moderate number							Moderate number						
Mar	Throat	Moderate number							Moderate number						
Noo	Throat	Moderate number							Moderate number						
Bra	Throat	Moderate number							Moderate number						
Kre	Nose	Moderate number							Moderate number						
Qui	Nose	Moderate number	+	+	0	+	+	+	Moderate number						
All	Throat	Moderate number	0	+	+	+	+	+	Moderate number	0	+	+	+	+	+
Kre	Rt. antrum	Few							Few						
Sea	Nose	Few							Few						
Bar	Nose	Few	+	0	+	0	0	0	Few	+	0	+	0	0	0
Bla	Nose	Occasional							Occasional						
Rod	Nose	Occasional							Occasional						
Don	Nose	Occasional							Occasional	+	0	+	+	+	+
38 patients		0							0						
Rad	Throat	Few			Yeast				0						
Bea	Tooth 1	Moderate number			Enterococci				Moderate number			Enterococci			
	Tooth 2	Moderate number			Enterococci				Moderate number			Enterococci			
Den	Nose	Few	0	0	0	0	0	0	Occasional	+	0	0	+	0	+

Den	Nose	Few	0	0	0	0	0	0	Occasional	+	0	0	+	0	+
War	Nose	0							Overgrown by spreader						
Sch	Throat	Moderate number			Yeast				Moderate number			Yeast			
Bat	Bile	Few			Enterococci				Few			Enterococci			
Mos	Throat	0							Many			Enterococci			
Sch	Nose	0							Few	0	0	0	0	0	0
Tit	Throat	Few			Yeast				Occasional			Yeast			
Lap	Nose	Many			B. coli				Many			B. coli			
Hol	Furuncle	Occasional	0	+	0	0	+	+	Occasional	0	+	0	0	0	0
Cultures which grew *slightly* better on phenol red mannitol agar: 23 (20.7%)																	
Ber	Nose	Many	0	0	0	+	+	+	Many						
Dou	Throat	Moderate number							Many						
Sie	Nose	Moderate number							Many						
Hun	Urine	Moderate number	+	+	+	0	+	+	Many						
Gam	Nose	Moderate number	+	0	±	+	+	+	Many	+	0	+	+	+	+
Wac	Nose	Moderate number							Many						
Mos	Nose	Moderate number	0	0	0	+	+	+	Many	0	0	+	+	+	+
Wac	Nose	Moderate number							Many						
Bea	Nose	Few	0	+	+	+	+	+	Moderate number	0	+	+	+	+	+
Bur	Nose	Few							Moderate number						
Bat	Nose	Few							Moderate number						
Opd	Nose	Few							Moderate number						
Kre	Left antrum	Occasional							Few						
Car	Nose	Occasional							Few						
Den	Throat	Occasional							Few						

* Order of reactions: P, pigment; H, hemolysis; C, coagulase; V, crystal violet agar; B, bromthymol blue agar, and M, mannitol agar fermentation. When all reactions were positive, they were not recorded in the tables.

TABLE 1—*Concluded*

PATIENT	SOURCE	BROMTHYMOL BLUE AGAR							PHENOL RED MANNITOL AGAR						
		Number	P	H	C	V	B	M*	Number	P	H	C	V	B	M
Cultures which grew *slightly* better on phenol red mannitol agar: 23 (20.7%)—*Concluded*															
Fri	Nose	0							Occasional	+	+	0	0	0	0
Nau	Throat	0							Occasional						
Kre	Left tonsil	0							Occasional						
	Rt. tonsil	0							Occasional						
Hun	Sputum	Few				Yeast			Occasional, also yeast						
Qui	Gums	0							Occasional	+	+	0	+	+	+
Bur	Throat	0							2 colonies in 48 hours						
Wac	Throat	0							Occasional						
Cultures which grew *moderately* better on phenol red mannitol agar: 13 (11.7%)															
Hun	Nose	Few	+	+	0	+	+	+	Many						
Rad	Nose	Few	0	+	+	+	+	+	Many	0	+	+	0	+	+
Bur	Nose	Few	0	0	+	+	+	+	Many						
Car	Throat	Occasional							Moderate number						
Bur	Throat	Occasional	+	0	+	+	+	+	Moderate number						
Sea	Throat	Occasional							Moderate number						
Sie	Throat	Occasional	+	+	+	0	+	+	Moderate number						
Nau	Nose	2 colonies							Moderate number						
Bar	Throat	0							Few						
Kre	Tooth	0							Few						
	Sputum	0							Few						
Ket	Nose	Occasional	0	0	0	0	0	0	Few	+	0	+	+	0	+
Lap	Throat	Occasional	0	0	0	0	+	0	Moderate number						

Cultures which grew *definitely* better on phenol red mannitol agar: 9 (8.1%)

Dal	Throat	1 colony		Many	
Hun	Throat	Occasional		Many	
Whi	Throat	0		Moderate number	
Sie	Throat	0		Moderate number	
Bla	Throat	0		Moderate number	
Van	Throat	0		Moderate number	
Qui	Throat	0		Moderate number	+ + 0 + + +
Bat	Sputum	0		Moderate number	
Bal	Nose	0		Moderate number	0 + + 0 + +

Cultures which grew better on bromthymol blue agar: 1 (0.9%)

Col	Throat	Few		0	

Crude cultures suspected of containing pathogenic staphylococci were plated on Bacto phenol red mannitol agar and incubated overnight. Acid-producing colonies were isolated and tested. Most of them proved to be pathogenic types, the results being qualitatively similar to those obtained with bromthymol blue agar. However, many phenol red mannitol agar plates showed better growth of in vitro positive staphylococci than the corresponding bromthymol blue agar plates. An occasional bromthymol blue agar plate gave better results. This suggested that both media should be used to complement each other.

Because they contain a large number of different bacteria, cultures from the nose, throat, sputum and gum margins of patients with chronic infections serve as a severe test of the efficiency of a medium for the isolation of pathogenic staphylococci. A series of 111 samples was inoculated on both media, spread by means of glass spreaders and incubated. Acid-producing colonies could be detected on phenol red mannitol agar within 18 to 24 hours. Occasionally the yellow zone was not apparent until later, while in other cultures the initial yellow zones disappeared after 24 to 48 hours incubation. Bromthymol blue agar plates required 36 to 48 hours incubation.

Typical or suspicious colonies were isolated from both media and tested by the following methods:

Pigment: Incubate on proteose lactose agar 48 hours. Scrape a loopful from the plate and examine it in a good light. Orange, lemon yellow, yellow and light yellow are considered positive (+) while cream, white and doubtful colors are considered negative (0).

Hemolysis: Spread a minute amount of culture on rabbit blood agar plates and incubate overnight. If the colonies have varying degrees of hemolysis, separate the hemolytic types and test again after the variants have been purified. A wide or moderate hemolytic zone is considered positive (+), while slight hemolysis or none at all is considered negative (0).

Coagulase: Mix 1 loopful of the culture from solid medium with 0.5 cc. of fresh human or rabbit plasma. Shake thoroughly and let stand. At the end of 3 hours, and again overnight, tilt the tube to a horizontal position and note the presence of a solid clot or a softer jelly-like mass which may appear as a swollen tapioca grain rising slightly above the surface, or as an opaque disc. In these instances the result is considered positive (+).

Crystal violet agar: Take a loopful of the culture from solid medium and make

a streak about 2 cm. long on a plate of crystal violet agar. Incubate 36 hours and observe the color of the growth. Violet or orange growths are considered positive (+), while white or pale violet growths are considered negative (0). Some of the white growths may have violet borders. Some non-pathogenic strains may fail to grow.

Bromthymol blue agar: Spread a loopful of the culture over the surface of a plate of bromthymol blue agar and incubate 36 to 48 hours. Strains producing luxuriant growths are considered positive (+) while those producing poor or no growth are considered negative (0).

TABLE 2

COMPARISON OF THE ADVANTAGES OF BROMTHYMOL BLUE AGAR AND PHENOL RED MANNITOL AGAR FOR THE ISOLATION OF PATHOGENIC STAPHYLOCOCCI

	BROMTHYMOL BLUE AGAR	PHENOL RED MANNITOL AGAR
Incubation	Requires 36 to 48 hours	Overnight usually sufficient
Inhibition	95% non-pathogenic staphylococci inhibited 37.7% pathogenic staphylococci slightly inhibited 21.3% pathogenic staphylococci moderately inhibited 14.8% pathogenic staphylococci markedly inhibited	No inhibition of staphylococci
Selectivity	Good. Inhibits most other bacteria	No inhibition of other bacteria
Value for isolation	Particularly useful when growth is heavy	Difficult to select colonies from crowded plate, due to (a) presence of contaminants and (b) the yellow color "runs"
Recovery advantage	Occasionally a pathogenic staphylococcus will grow on this medium but will not ferment mannitol	This medium is extremely sensitive to mannitol-fermenting colonies and will detect them even when numerous other bacteria are present
Interfering bacteria	Yeasts, enterococci, coli-aerogenes	Yeasts, enterococci, coli-aerogenes, any mannitol-fermenting organism

Mannitol fermentation: Spread the culture on a petri dish of Difco phenol red mannitol agar and incubate overnight. Colonies surrounded by yellow zones are mannitol positive (+), while those with no yellow zones are negative (0).

The criteria of Chapman, Berens, Peters and Curcio[3] were used in interpreting the results of hemolysis and coagulase tests which, in most cases, were parallel with the other in vitro and in vivo tests. Any coagulating strain, also

hemolytic aureus strains (i.e. pigment, hemolysis and coagulase tests of +++, 0++, +0+, 00+ and ++0) were considered positive. A few strains which were negative to the hemolysis and coagulase tests (i.e. 000, +00 or 0+0) gave positive reactions to the crystal violet agar, bromthymol blue agar and mannitol

TABLE 3

SUGGESTED SCHEME FOR THE ISOLATION AND TESTING OF PATHOGENIC TYPES OF STAPHYLOCOCCI

Spread the culture on

Bromthymol blue agar

Incubate 36 to 48 hours. If no growth, discard.

If typical colonies are present, save them in case pathogenic types are not isolated from phenol red mannitol agar.

Phenol red mannitol agar

Incubate overnight. If there are no colonies surrounded by yellow zones, incubate longer.

Typical yellow-zoned colonies are probably pathogenic staphylococci.

For rapid test, add 1 loopful of the culture mass to 0.5 cc. of human plasma. Shake thoroughly and incubate. Examine in 3 and 12 hours for the presence of a fibrin clot. If positive, the strain is probably pathogenic.

Plate the culture on:

Rabbit blood agar, incubate overnight, and examine for hemolysis.

Proteose lactose agar or Loeffler's medium, incubate 36 hours, and examine for pigment. The growth may also be used to confirm the coagulase reaction of the crude culture.

Crystal violet agar, incubate 36 hours, and examine for color of growth.

Bromthymol blue agar, incubate 36 hours, and examine for luxuriance of growth.

Phenol red mannitol agar, incubate overnight, and examine for acid production.

Animal inoculation and other tests may be used for confirmation of these in vitro tests.

The above scheme does not include tests for particular toxins, such as exotoxin, necrotoxin, gastroenterotoxin, leucocidin, acute killing toxin, beta hemolysin, etc.

fermentation tests. For reasons which will be discussed elsewhere[2] they were considered only feebly pathogenic. As a rule non-pathogenic strains reacted negatively to all six tests.

The results of comparison of the 111 cultures are detailed in table 1. For

simplicity, the reactions to the 6 tests just described were recorded only when the strain did not react positively to all. It will be seen that both media gave similar results in 58.5 per cent of the cultures. Phenol red mannitol agar gave slightly better results in 20.7 per cent, moderately better in 11.7 per cent, and definitely better in 8.1 per cent of the cultures. Bromthymol blue agar gave better results in 1 culture (0.9 per cent).

During the course of these studies, it was noted that, in addition to their selective ability, both media had peculiarities which influenced their usefulness. From the summary of these factors in table 2 it is evident that, for best results, a combination of both methods should be used to isolate the maximum number of pathogenic staphylococci.

Phenol red mannitol agar is useful for rapid isolation of probable pathogenic staphylococci, 98.3 per cent of the colonies isolated proving to be of the pathogenic type. Bromthymol blue agar is more suitable when a large number of different bacteria are present or in those occasional instances where a pathogenic staphylococcus does not ferment mannitol. The chance of missing a pathogenic strain by the combined use of these two media is in proportion to the number of strains that fail to grow on bromthymol blue agar and fail to ferment mannitol. Of 367 hemolysis-coagulase-positive strains from pathologic sources, only 2 reacted negatively to these 2 tests.

For best results it is suggested that the scheme outlined in table 3 be used for the isolation of probable pathogenic staphylococci.

CONCLUSION

Probable pathogenic staphylococci may be isolated from mixed cultures by using bromthymol blue agar and phenol red mannitol agar for initial isolation.

REFERENCES

(1) Chapman, G. H., Lieb, C. W., Berens, C. and Curcio, L. G.: The isolation of probable pathogenic staphylococci. Jour. Bact. **33:** 533–543. 1937.

(2) Chapman, G. H., Berens, C., Nilson, E. L. and Curcio, L. G.: Differentiation of pathogenic staphylococci from non-pathogenic types. Jour. Bact. In press.

(3) Chapman, G. H., Berens, C., Peters, A. and Curcio, L. G.: Coagulase and hemolysin tests as measures of the pathogenicity of staphylococci. Jour. Bact. **28:** 343–363. 1934.

THE DETERMINATION OF BLOOD CREATINE USING 3,5-DINITROBENZOIC ACID AS A COLOR REAGENT*

JEROME E. ANDES

From the Department of Pathology, West Virginia University Medical School, Morgantown

Three different groups of investigators[1,2,3] working independently, have recently demonstrated that the sodium salt of 3,5-dinitrobenzoic acid produces, with creatinine in a basic solution, a purple color sufficiently proportional to the concentration to be useful in its determination. The fact that the values for blood creatinine, as obtained with the new reagent, were found to be somewhat lower than those obtained by picric acid, has led me to apply the reagent to the determination of blood creatine, and to compare the results with those obtained with the picric acid method.

METHODS

Precipitation of Blood Proteins

The blood proteins were precipitated with tungstic acid according to the procedure of Folin and Wu,[4] with the exception that the acid was added before the tungstate.

Procedure. Add 1 volume of blood to 7 volumes of water and allow to stand 4–5 minutes to lake the red cells. Then add one volume of ⅔ normal sulfuric acid, shake, and add one volume of 10 per cent sodium tungstate. The mixture can be filtered (or centrifuged) at once, and yields a clear filtrate that can be autoclaved with HCl without charring.

Determination of Preformed and Total Creatinine (Creatine) with Picric Acid

The procedure of Folin and Wu[4] was followed with only a few minor variations. In determining the total creatinine (creatine), 20 cc. of the filtrate in a 25 cc. volumetric flask was treated with 0.4 cc. of concentrated HCl, autoclaved 30 minutes at 120°, neutralized and made up to volume. The creatinine was determined on 10 cc. of this solution using the method for preformed creatinine and employing a suitable standard (3.0 mg. per 100 cc. with normal cases).

---

* Received for publication June 1st, 1937.

Creatine was calculated by subtracting the preformed from the total creatinine, and multiplying the result by 1.16.

Determination of Preformed and Total Creatinine (Creatine), Employing 3,5-Dinitrobenzoic Acid as a Color Reagent

Preformed Creatinine. The method used is a modification of that of Langley and Evans[2]. It consists chiefly of a direct comparison of the color produced by the reagent with blood filtrate and pure creatinine solution.

Procedure. Pipette 10 cc. of blood filtrate into a small flask, and add 10 per cent NaOH, drop by drop, until red litmus is just turned blue (only *one* drop is necessary if the precipitating agents are prepared and added correctly.) Prepare a creatinine standard by measuring 0.375 cc. of the standard creatinine solution by measuring 0.375 cc. of the standard creatinine solution (equivalent to 0.01125 mgm. of creatinine)* into a small flask, making up to 10 cc. with water, and neutralizing as with the filtrate. Again only one drop of 10 per cent NaOH is required. The standard thus prepared is equivalent to 1.125 mgm. of creatinine per 100 cc. of blood.

To both the standard and unknown add 3 cc. of the 3,5-dinitrobenzoic acid reagent,† and 0.5 cc. of 10 per cent NaOH. Allow to stand 8–10 minutes and compare the colors in a colorimeter. If the reading of the unknown is not close to that of the standard, a graph had best be used.

---

* The stock creatinine standard is the same as that used for the picric acid method. It contains 3.00 mgm. creatinine per 100 cc. of N/10 HCl. For convenience in making the normal standard, the stock standard is diluted 1:4 with distilled water, and 1.5 cc. of this dilution used instead of 0.375 cc. of the stock solution. The diluted standard appears to keep indefinitely.

With blood samples showing a creatinine concentration above normal, a standard must be made to approximate that of the unknown. Since very little color is given by the reagent itself, the concentration of creatinine with higher standards is practically proportional to the color produced; hence standards containing 0.01125, 0.03, 0.06, and 0.15 mgm. of creatinine (0.375, 1.0, 2.0, and 5.0 cc. respectively of the regular creatinine standard) in a volume of 10 cc. will cover most of the concentrations that are found in human blood.

† The 3,5-dinitrobenzoic acid must be purified before using. This is easily carried out by the method of Langley and Evans (2). Dissolve 50 gm. of the acid in 100 cc. of boiling 80 per cent alcohol, filter and allow to stand at least two hours at about 3°C. Filter off the crystals formed, wash with 50 per cent alcohol, and dry at 100°C. for several hours.

Preparation of Reagent. Add 20 gm. of the above purified acid to 150 cc. of water and then add 50 cc. of 10 per cent Na_2CO_3. Warm (do not boil) until solution takes place. The resultant reagent should be a *very pale* yellow color; if such is not the case, the acid must be recrystallized.

Calculation.

$$\frac{S}{R} \times S_1 \times 100 = \text{mgm. Creatinine per 100 cc. of Blood}$$

Where S = Reading of Standard,
R = Reading of Unknown, and
S_1 = mgm. of Creatinine in Standard (0.01125 if the normal standard is used).

TABLE 1

COMPARISON OF THE TWO METHODS WITH PURE CREATINE SOLUTIONS

All figures (except percentages) in mgm. per 100 cc.

CREATINE IN SAMPLE	PICRIC ACID METHOD			3,5-DINITROBENZOIC ACID METHOD		
	Detn. as creatinine	× 1.16 (creatine)	Per cent recovery	Detn. as creatinine	× 1.16 (creatine)	Per cent recovery
3.50	3.02	3.50	100.0	2.99	3.46	99.0
3.50	2.98	3.46	99.0	3.02	3.50	100.0
3.50	3.02	3.50	100.0	2.99	3.46	99.0
3.50	2.96	3.43	98.1	3.03	3.51	100.3
3.30	2.85	3.31	100.3	2.84	3.29	99.7
3.30	2.83	3.29	99.7	2.85	3.30	100.0
3.30	2.84	3.30	100.0	2.80	3.25	98.5
3.30	2.82	3.27	99.1	2.85	3.30	100.0
1.80	1.53	1.78	99.0	1.56	1.81	100.5
1.80	1.55	1.79	99.5	1.55	1.80	100.0
1.80	1.55	1.79	99.5	1.53	1.78	98.8
1.80	1.56	1.81	100.5	1.55	1.80	100.0
1.80	1.55	1.80	100.0	1.56	1.81	100.5
2.50	2.08	2.41	96.5	2.13	2.47	98.8
2.50	2.16	2.52	100.8	2.19	2.53	101.0
2.50	2.16	2.52	100.8	2.18	2.52	100.8
2.50	2.14	2.48	99.2	2.19	2.53	101.1
3.75	3.23	3.75	100.0	3.23	3.75	100.0
3.75	3.25	3.77	100.5	3.22	3.74	99.8
3.75	3.15	3.68	98.0	3.26	3.78	100.8
3.75	3.27	3.79	101.0	3.24	3.76	100.2
Average recovery.....			99.7			100.0

Creatine. The creatinine in the blood filtrate is determined before and after autoclaving with HCl (preformed and total creatinine respectively), and the creatine calculated from the difference.

Procedure for Total Creatinine. Measure 20 cc. of blood filtrate into a 25 cc. volumetric flask, add 0.4 cc. of concentrated HCl, and autoclave for 30 minutes at 120°C. (20 pounds pressure). Cool, carefully neutralize with 10 per cent NaOH using litmus as an indicator, and make up to volume with

water. Determine the creatinine in 10 cc. of this solution in the same manner as for preformed creatinine. The standard for normal blood should contain 0.03 mgm. of creatinine (1 cc. of the stock standard) per 10 cc. of volume. The acid in the standard must be neutralized with NaOH before it is made up to volume.

Calculation:

$$\frac{S}{R} \times S_1 \times 100 = \text{mgm. Total Creatinine per 100 cc. of Blood}$$

Where S = Reading of Standard,
R = Reading of Unknown, and
S_1 = mgm. of creatinine in standard (0.03).

TABLE 2

RECOVERY OF CREATINE ADDED TO BLOOD FILTRATE

(New method)

TOTAL CREATININE IN FILTRATE*	CREATINE ADDED*	CREATINE RECOVERED*	PER CENT RECOVERY
4.27	37.5	36.9	98.5
4.39	37.5	37.2	100.3
4.29	30.0	30.1	100.3
3.96	30.0	30.0	100.0
4.50	30.0	30.3	100.9
4.22	30.0	31.2	103.8
3.83	30.0	30.4	101.2
3.65	30.0	31.5	105.0
3.95	15.0	15.04	100.3
4.06	15.0	14.88	99.1
4.37	15.0	14.94	99.7
3.88	15.0	14.84	98.8
Average			100.7

* Figures in mgm. per 100 cc. of filtrate.

Calculation of Creatine: Determine the preformed creatinine as described previously. Subtract this value from that obtained for the total creatinine (above), and multiply by 1.16.

EXPERIMENTAL

In Table 1 is shown a comparison of the amount of creatinine produced from the autoclaving of creatine* in acid solution, with

* The creatine used in this work was obtained from the Eastman Kodak Company. After recrystallization by the procedure outlined by Folin[5], it was found to be at least 99.8 per cent pure.

TABLE 3

CREATININE AND CREATINE IN NORMAL WHOLE BLOOD WITH THE PICRIC ACID AND THE 3,5-DINITROBENZOIC ACID METHODS

(Figures in mgm. per 100 cc.)

CASE NO.	PICRIC ACID METHOD			DINITROBENZOIC ACID METHOD		
	Preformed creatinine	Total creatinine	Creatine	Preformed creatinine	Total creatinine	Creatine
1	1.4	4.5	3.6	1.1	4.4	3.8
2	1.5	4.7	3.6	1.0	4.3	3.8
3	1.2	4.2	3.4	1.1	4.3	3.8
4	1.2	3.8	3.0	1.0	3.8	3.3
5	1.4	4.1	3.2	1.1	4.0	3.3
6	1.6	4.7	3.6	1.2	4.7	4.0
7	1.4	3.7	2.7	1.4	3.7	2.6
8	1.2	3.5	2.6	1.3	3.3	2.3
9	1.4	3.9	2.8	1.0	3.6	3.1
10	1.3	3.6	2.7	0.9	3.3	2.7
11	1.5	3.9	2.7	1.3	3.5	2.6
12	1.2	4.3	3.5	1.3	4.7	3.8
13	1.2	3.8	2.9	1.3	4.2	3.3
14	1.0	3.4	2.7	1.1	3.7	3.0
15	1.2	3.8	3.0	1.2	4.0	3.2
16	1.5	4.2	3.1	1.4	4.7	3.9
17	1.4	3.8	2.7	1.2	3.8	3.0
18	1.4	4.0	3.1	1.2	4.1	3.4
19	1.3	3.8	2.9	1.2	4.0	3.1
20	1.3	3.8	2.9	1.2	3.9	3.1
21	1.4	4.1	3.2	1.3	4.0	3.4
22	1.2	3.6	2.7	1.2	3.9	3.1
23	1.4	4.0	3.0	1.2	4.2	3.6
24	1.3	4.1	3.2	1.1	4.3	3.5
25	1.4	3.9	2.9	1.3	4.5	3.7
26	1.2	4.4	3.7	1.3	4.8	4.2
27	1.3	3.8	2.9	1.1	3.9	3.2
28	1.4	4.1	3.1	1.2	4.1	3.4
29	1.4	4.6	3.7	1.2	4.6	4.0
30	1.4	4.6	3.7	1.1	4.4	3.8
High.....	1.6	4.7	3.7	1.4	4.8	4.2
Low.....	1.0	3.4	2.6	0.9	3.3	2.3
Average...	1.34	4.01	3.10	1.19	4.10	3.37

both color reagents. Complete recovery was obtained in both cases. In Table 2 is shown the recovery of creatine added to blood filtrate, using the new color reagent. Again complete

recovery was obtained, indicating that *any creatine actually present* in a blood filtrate will be determined with *either* reagent.

In Table 3 is shown the values for preformed creatinine, total creatinine, and creatine (the latter calculated as creatine), in the blood of 30 normal individuals. In a study of the results, it will be noted that the new reagent gives values for preformed creatinine about 0.2 mg. lower than the picric acid method; however, with creatine the average value is about 0.3 mgm. higher. The average value for total creatinine is practically the same for both reagents, namely 4.0 and 4.1 mgm. per 100 cc. of blood. The range of concentrations for both creatine and creatinine are about the same for both methods.

DISCUSSION OF RESULTS

The values obtained for preformed creatinine (using picric acid) are essentially the same as the figures reported in the literature. The preformed creatinine, as determined by 3,5-dinitrobenzoic acid is somewhat higher than that reported by Langley and Evans[2]. This may be due to several factors. The color produced by the filtrate is of a different shade (more yellow) than that of the standard, and accurate color comparisons are somewhat difficult. Also, the amount of base added in the determination makes a distinct difference in the values obtained. I found it necessary to bring the standard and filtrate approximately to the same pH (see method), in order to obtain reproducible results. My figures, with the new reagent, average less than 0.2 mg. below those obtained with picric acid.

The figures for creatine, as determined with picric acid, are below most of the values reported (using Folin's or similar methods). The figures given by Folin and Wu[4] are between 4.1 and 6.4 mgm. per 100 cc. (calculated as creatine); the values reported by other workers are mostly between 3 and 7 mgm. I believe the difference is due to the *charring* that so frequently occurs, when the filtrate (prepared in the standard way) is autoclaved with acid. In fact, Bohn, Friedsam, and Hahn[6] state that the hydrolysis of the creatine must be carried out at a temperature below 60° to prevent this charring. They also state

that the true creatine values are about one-third that of the accepted ones. My values are about one-half of the accepted figures.

The creatine, as determined by 3,5-dinitrobenzoic acid, is slightly higher than the values obtained with picric acid (the reverse of the case with preformed creatinine). However, the total creatinine is almost the same with both methods. This seems to indicate that, although the preformed chromogenic substances in blood (creatinine or creatinine-like) react to a different degree with the two reagents, after autoclaving these substances are all converted into creatine (or very similar compounds).

The adoption of the 3,5-dinitrobenzoic acid methods (for routine chemical analyses) for the determination of creatinine and creatine in blood, is not recommended at the present. The purification of the color reagent must be carefully carried out or the results are wholly unsatisfactory. Also, the degree of acidity of the unknown and standard must be carefully regulated while the color is developing, or the results are not reliable. In addition, the new reagent is quite expensive as compared to picric acid. The only real advantageous point in using the new reagent is the greater ease with which the colors can be compared.

SUMMARY

1. The precipitation of blood proteins by tungstic acid (using the Folin-Wu reagents) in the reverse order, gives a filtrate that remains colorless after autoclaving with hydrochloric acid.

2. The use of 3,5-dinitrobenzoic acid as a color reagent for the determination of creatinine, gives colors more easily compared than with picric acid.

3. A method is outlined for the estimation of blood creatine using the above reagent which gives 100 per cent recovery of added creatine.

4. In a study of 30 normal bloods, using both picric acid and 3,5-dinitrobenzoic acid as color reagents, the latter showed slightly lower values for preformed creatinine and slightly higher values for creatine. Both methods gave a similar average value for total creatinine.

5. The results obtained for creatine with both methods are considerably lower than most of those reported by other workers (using similar procedures). This is attributed to the manner of preparing the blood filtrate.

REFERENCES

(1) Benedict, S. R. and Behre, J. A.: Some applications of a new color reaction for creatinine. J. Biol. Chem., **114:** 515. 1936.

(2) Langley, W. D. and Evans, M.: The determination of creatinine with sodium 3:5-dinitrobenzoate. J. Biol. Chem., **115:** 333. 1936.

(3) Bolliger, A.: A new color reaction for creatinine. J. Proc. Roy. Soc. N.S. Wales, **69:** 224. 1936.

(4) Folin, O. and Wu, H.: A sysgem of blood analysis. J. Biol. Chem., **38:** 81. 1919.

(5) Folin, O.: On the preparation of creatine, creatinine and standard creatinine solutions. J. Biol. Chem., **17:** 463. 1914.

(6) Bohn, H., Friedsam, A. and Hahn, F.: True and apparent creatine and creatinine of blood and urine. Zentral. inn. Med., No. **22:** 465. 1935.

MOLASSES AGAR FOR THE ISOLATION AND CULTIVATION OF YEASTS AND MOLDS

SOL HABERMAN

From the Botany-Bacteriology Department, University of Texas, Austin, Texas

A nutrient agar base (beef extract 0.3 per cent, peptone 0.5 per cent, agar 2 per cent) to which the desired amount of "sorghum" molasses has been added furnishes an efficient, cheap and simplified medium for the isolation and cultivation of yeasts and molds.

The amount of molasses to be added to the nutrient agar base is determined by the purpose for which the medium is to be used. When intended for the isolation of yeasts and molds, molasses is added to make eight per cent by volume. When the medium is to be used for the cultivation of yeasts and molds the molasses content should be two per cent by volume, the organisms remaining viable with this concentration longer than when greater quantities of molasses are used.

Preliminary studies[1] have shown that when varying quantities of molasses are added to Difco nutrient agar having a pH of 6.9 a corresponding variation occurred in the pH of the medium after sterilization. Thus, after autoclaving 20 minutes at 15 lbs. pressure, the pH of varying percentages of molasses agar was as follows: 1 per cent, 6.6; 2 per cent 6.52; 4 per cent, 6.35, and 8 per cent, 6.08. When the duration of autoclaving was increased a marked shift toward the acid range took place.

An eight per cent molasses agar is more suitable for the isolation of molds because of its lower pH which tends to lessen bacterial contamination, and, for this reason the pH may be advantageously brought to 5.5 by the addition of N/1 hydrochloric acid.

In fourteen patients presenting skin infections of possible fungus origin, dermal scrapings were planted on 8 per cent molasses agar. After 72 hours incubation monilia were recovered from six cases, trichophyton in one case, and epidermophyton in one, the cultures in the remainder (6 cases) being negative.

The monilia recovered, together with stock strains of M. albicans and M. psilosis, were plated on one, two, four and 8

per cent molasses agar, on honey agar of equivalent percentages by volume, and on Difco Saboraud agar. After 48 hours at 37°C, the greatest growth occurred on the 2 per cent molasses agar, the growth characteristics on this medium being typical. This was also true of the trichophyton and epidermophyton cultures on this medium.

Similar comparisons were made with soil fungi using malt agar, Waksman's agar, and 8 per cent molasses agar. Both stock cultures and isolated strains were compared on these media, using Gilman and Abbott's key to the soil fungi.[2]

One gram amounts from each of two soil samples and one sample of stable manure were diluted 1:10,000, 1:100,000, and 1:1,000,000 and plated on each of the media above mentioned and the plates observed at twenty-four hour intervals for bacterial and fungus colonies.

Bacteria were present on all the plates, the largest number of colonies being found on the molasses agar at pH 6.08. When the pH of this medium was brought to 5.5 by the addition of N/1 hydrochloric acid the results were comparable to those obtained with the other media.

Although the bacterial colonies were numerous on the pH 6.08 molasses agar, they were soon overgrown by the fungi, the number and variety of which was greater than obtained on malt agar or Waksman's agar.

In pure culture the growth of these fungi, as also of stock strains, was more rapid on the molasses agar than on the other media with which it was compared. Growth characteristics were typical and spore heads and other reproductive structures in most cases appeared earlier. On this medium Rhizopus nigricans and Absodia of known plus and minus strains formed zygospores profusely.

The fungi thus studied included Rihzopus, Absidia, Penicillium, Aspergillus, Mucor, Alternaria, and Cunninghamella. Similar results were also obtained with Saccharomyces cerevisiae.

From these studies it appears that molasses agar is a suitable medium for yeasts and molds, 8 per cent being suitable for isolation media, and 2 per cent most suitable for cultivation.

While different brands of "sorghum" molasses vary in nitrogen

and sugar content, this variation does not appear to influence appreciably the results obtained with this medium.

In all these studies crude "sorghum,' molasses was used, suggesting that the results obtained may not be due entirely to utilizable foods.

REFERENCES

(1) KEMP, H. A. AND HABERMAN, S.: Field and Laboratory, **3**: 5-6. 1934.
(2) GILMAN, J. C., AND ABBOTT, E. V.: Iowa State College Jour. Sc., **1**: 225. 1927.

THE DETERMINATION OF BLOOD PLASMA AND SPINAL FLUID PROTEINS*

GEORGE W. JOHNSTON AND R. B. GIBSON

From the Pathological Chemistry Laboratory, State University of Iowa General Hospital, Iowa City, Iowa

Since the introduction of a method for the colorimetric determination of blood plasma proteins by Wu[10], various modifications have been proposed. Greenberg[5] introduced the use of sodium sulfate as the globulin precipitant (according to the method of Howe[7] and the improved phenol reagent of Folin and Ciocalteu[4]). Andersch and Gibson[1] found that the color produced with the phenol reagent was greatly intensified and became constant if the proteins were heated in an alkaline solution prior to the addition of the reagent. They proposed a method for fractionating the proteins, using ammonium sulfate and heating with alkali. Certain criticisms of the method are valid: the high values for fibrinogen and the low values for albumin. Greenberg[6] adapted the Andersch-Gibson method of heating the proteins in alkaline solution to his earlier procedure and found that the 30 minute period as proposed could safely be reduced to 10 minutes. The method of Minot and Keller[9] is essentially the same as Greenberg's. However, the tyrosine factors they found were slightly higher than those reported by the previous author which they believed to be probably due to heating the proteins

* Received for publication April 20th, 1937.

with a slightly less concentrated solution of alkali. Cameron et al.[2] used a technique similar to that employed by Andersch and Gibson with the exceptions of using Wu's improved phenol reagent,[1] separating the fibrin according to the method of Cullen and Van Slyke,[3] and applying corrections to the colorimeter readings.

In seeking a method suitable for the routine determination of blood plasma and spinal fluid proteins we found that those already in use either employed a technic that was too involved, gave inconstant results, or too long a time was required in carrying out the determinations. In the methods presented in this paper we have made an attempt to combine the better features of all the previous methods, relative to the constancy of results, ease of manipulation, and the time necessary to carry out the determinations.

METHODS

Blood Plasma Proteins

A rack of 15 cc. conical centrifuge tubes, a container of distilled water, and a 30 per cent solution of anhydrous sodium sulfate are warmed to approximately 37°C., in an incubator.

Fibrinogen. Precipitated with 11.25 per cent Na_2SO_4.

To 1 cc. of plasma in a 15-cc. centrifuge tube is added 1.5 cc. of distilled water and 1.5 cc. of 30 per cent sodium sulfate. Mix by inversion and place in incubator at approximately 37°C., for at least 10 minutes. Centrifuge, pour off the supernatant liquid into another tube and save. Wash the precipitate with 11.25 per cent sodium sulfate (2.5 cc. of water and 1.5 cc. of 30 per cent sodium sulfate). Return to the incubator for about 10 minutes, then centrifuge and pour off the wash solution (discard). Wipe the lip of the tube free of any adhering liquid with a piece of gauze or filter paper. Retain the fibrinogen precipitate.

Globulin. Precipitated with 22.5 per cent sodium sulfate (determined by difference).

To 2 cc. of the supernatant liquid (from above) in a centrifuge or test tube add 3 cc. of a 30 per cent solution of sodium sulfate, mix by inversion and place in the incubator at about 37°C., for at least 10 minutes. Filter through a pleated paper in the incubator and return the filtrate through the same paper until it becomes clear.

Albumin. To 2 cc. of the globulin filtrate in a 15-cc. centrifuge tube, add 3 cc. of distilled water and 1 cc. of 20 per cent trichloroacetic acid. Mix by in-

version, allow a few minutes for the albumin precipitate to flocculate and centrifuge. Pour off the supernatant liquid and wipe the lip of the tube with a piece of gauze or filter paper. Retain the precipitated albumin.

Total Protein. To 0.5 cc. of 10 per cent sodium hydroxide in a 15-cc. centrifuge tube, add 0.1 cc. of plasma from a pipette graduated to contain 0.1 cc. Wash the lumen of the pipette free of plasma by drawing up and discharging the alkali solution 2 or 3 times.

Add 0.5 cc. of 10 per cent sodium hydroxide to the tubes containing the albumin and fibrinogen precipitates. Heat all of the tubes (including total protein) in a boiling water bath for 10 minutes. Agitate after 3–4 minutes heating to make sure that all of the protein is in solution.

Add 7.5 cc. of distilled water to each tube and 6.0 cc. to another tube containing 2 cc. of a standard tyrosine solution (20 mgm.:100 cc. in approximately N/10 HCl). Add 1 cc. of Folin-Ciocalteu color reagent and 3 cc. of a saturated solution of sodium carbonate to each tube. Mix by inversion, allow 10 minutes for color to develop, compare in colorimeter with standard set at 10 mm.

Calculations. The tyrosine equivalents were found to be:

1 mg. of tyrosine = 10.3 ± 0.2 mgm. of fibrinogen
1 mg. of tyrosine = 11.1 ± 0.2 mgm. albumin
1 mg. of tyrosine = 11.4 ± 0.0 mgm. total protein

Fibrinogen. $\frac{10}{R} \times \frac{0.4}{1} \times 100 \times 10.3 = \frac{4120}{R}$ = mgm. fibrinogen/100 cc.

or $\frac{4.1}{R}$ = grams per cent of fibrinogen.

Albumin. $\frac{10}{R} \times \frac{0.4}{0.2} \times 100 \times 11.1 = \frac{22200}{R}$ = mgm. of albumin/100 cc.

or $\frac{22.2}{R}$ = grams per cent of albumin.

Total Protein. $\frac{10}{R} \times \frac{0.4}{0.1} \times 100 \times 11.4 = \frac{45600}{R}$ = mgm. protein/100 cc.

or $\frac{45.6}{R}$ = grams per cent of total protein.

Globulin. (estimated by difference). Total protein − (fibrinogen + albumin) = Globulin.

Total Protein in Spinal Fluid

To 2 cc. of spinal fluid in a 15-cc. centrifuge tube, add 3 cc. of distilled water and 1 cc. of 20 per cent trichloroacetic acid. Mix by inversion, allow a few minutes for the protein to floc and then centrifuge. Pour off the supernatant liquid (discard) and free the lip of the tube of any adhering liquid with a piece of gauze or filter paper. Add 0.25 cc. of 10 per cent sodium hydroxide and heat in a boiling water bath for 10 minutes. Add 3.75 cc. of distilled water, 0.5 cc. of Folin-Ciocalteu color reagent and 1.5 cc. of a saturated solution of

sodium carbonate. Mix by inversion, allow 10 minutes for color to develop and compare with the nearest standard in the colorimeter with the standard set at 10 mm.

Preparation of Standards

Three standards should be made up as follows:

0.5 standard. To 0.5 cc. of standard tyrosine solution (20 mgm./100 cc. in approximately N/10 HCl), add 7.5 cc. of distilled water, 1 cc. of Folin-Ciocalteu color reagent and 3 cc. of a saturated solution of sodium carbonate.

1.0 standard. One cubic centimeter of the standard tyrosine solution, dilute to 8 cc. and treat as above.

2.0 standard. Two cubic centimeters of the standard tyrosine solution, dilute to 8 cc. and treat as above.

Calculations. The tyrosine factor found for total blood plasma protein was employed.

$$0.5 \text{ standard: } \frac{10}{R} \times \frac{0.1}{2} \times \frac{100}{2} \times 11.4 = \text{mgm. per cent of protein,}$$

$$\text{or } \frac{285}{R} = \text{mgm. per cent of protein.}$$

$$1.0 \text{ standard: } \frac{10}{R} \times \frac{0.2}{2} \times \frac{100}{2} \times 11.4 = \text{mgm. per cent of protein,}$$

$$\text{or } \frac{570}{R} = \text{mgm. per cent of protein.}$$

$$2.0 \text{ standard: } \frac{10}{R} \times \frac{0.4}{2} \times \frac{100}{2} \times 11.4 = \text{mgm. per cent of protein,}$$

$$\text{or } \frac{1140}{R} = \text{mgm. per cent of protein.}$$

EXPERIMENTAL

Determination of Tyrosine Factors

Macro-Kjeldahl nitrogen determinations were carried out on the blood plasma protein fractions after precipitation with sodium sulfate (according to the method of Howe[7, 8]). The nitrogen values were converted to protein by use of the commonly used factor 6.25. A series of three determinations in duplicate were carried out on mixed plasma.

Tyrosine was determined colorimetrically by the method described. Six tyrosine determinations were done in each instance and the average values were used. The tyrosine equivalents found are given in the method as outlined above.

Check on the Method

Since there has been considerable controversy in regard to the various procedures employed, we decided to check each step individually.

TABLE 1

TIME NECESSARY FOR THE FIBRINOGEN AND GLOBULIN FRACTIONS TO BE COMPLETELY SEPARATED AT SODIUM SULFATE CONCENTRATIONS OF 10.5 AND 22.5 PER CENT RESPECTIVELY, AT APPROXIMATELY 37°C.

TIME	FIBRINOGEN	GLOBULIN (BY DIFFERENCE)
	grams per cent	*grams per cent*
10 minutes	0.41	2.57
20 minutes	0.41	2.57
30 minutes	0.39	2.62
1 hour	0.40	2.65
2 hours	0.41	2.51
4 hours		2.68
16 hours		2.57

TABLE 2

TIME NECESSARY FOR THE COLOR DEVELOPMENT TO BECOME CONSTANT

	TIME	COLORIMETER READINGS			
		Fibrinogen	Albumin	Total protein	Spinal fluid protein
	minutes				
A	10		6.7	7.3	11.4
	20		6.7	7.4	11.3
	30		6.7	7.4	11.3
	45		6.7	7.3	
B	10	23.6	5.0	6.4	7.8
	20	23.6	5.0	6.4	7.8
	30	23.6	5.0	6.4	7.8
	60	23.6	5.2	6.4	
C	10	18.3	5.1	6.4	15.0
	20	18.5	5.1	6.4	15.0
	30	18.4	5.1	6.4	15.0
	60	18.5	5.1	6.4	
D	10	23.6	5.4	6.6	7.5
	20	23.6	5.4	6.6	7.5
	30	23.6	5.5	6.7	7.5
	60	24.4	5.5	6.6	

Maximum precipitation of the fibrinogen and globulin fractions at 37°C., occurred in as little time as ten minutes, as shown in table 1.

The color development was constant after standing for ten minutes at room temperature, as shown in table 2.

The concentrations of anhydrous sodium sulfate necessary to precipitate fibrinogen and globulin were checked in order to make sure that those given by

TABLE 3

PRECIPITATION OF PROTEIN FROM 1 CC. OF PLASMA WITH VARYING CONCENTRATIONS OF SODIUM SULFATE AT APPROXIMATELY 37°C.

SODIUM SULFATE	PROTEIN
grams per cent	*grams per cent*
9.37	0.45
9.75	0.48
10.12	0.52
10.50	0.58
10.87	0.54
11.25	0.58
11.62	0.66

TABLE 4

AMOUNT OF PROTEIN PRECIPITATED FROM FIBRINOGEN FILTRATE WITH VARYING CONCENTRATIONS OF SODIUM SULFATE AT APPROXIMATELY 37°C.

SODIUM SULFATE	PROTEIN IN PRECIPITATE BY DIFFERENCE	SODIUM SULFATE	PROTEIN IN PRECIPITATE BY DIFFERENCE
grams per cent	*grams per cent*	*grams per cent*	*grams per cent*
11.4	0.95	22.8	2.42
12.6	1.18	23.0	2.42
13.8	1.39	23.3	2.42
15.0	1.67	23.5	2.47
16.2	1.80	23.7	2.89
17.4	2.07	23.9	2.76
18.6	2.07	24.1	2.86
19.8	2.18	24.3	2.89
21.0	2.35	24.5	2.89
22.2	2.50	24.7	2.79
22.5	2.47	24.9	2.89

Howe[7, 8] were applicable to our modified procedure, as shown in tables 3 and 4 respectively.

It is seen from table 3 that approximately the same amount of protein is precipitated between sodium sulfate concentrations of 10.5 and 11.25 per cent. The upper limit of sodium sulfate concentration necessary for the complete precipitation of fibrinogen is taken as 11.25 per cent. Similarly (table 4) ap-

proximately the same amount of protein is precipitated between sodium sulfate concentrations of 22.2 per cent and 23.5 per cent. In order to simplify the technique a sodium sulfate concentration of 22.5 per cent is used for complete precipitation of the globulin fraction. These precipitations were carried out at 37°C.

In table 5 blood protein values determined by the Andersch-Gibson method are compared with values by the described modified procedure.

TABLE 5

COMPARISON OF VALUES OBTAINED WITH THE ANDERSCH-GIBSON METHOD AND THE DESCRIBED PROCEDURE

PROTEIN FRACTION	ANDERSCH-GIBSON METHOD	DESCRIBED PROCEDURE
	grams per cent	*grams per cent*
Fibrinogen	0.44	0.23
Globulin (by difference)	3.16	2.72
Albumin	3.07	4.29
Total protein	6.67	7.24

TABLE 6

BLOOD PROTEIN VALUES OF NORMAL INDIVIDUALS BETWEEN AGES OF 20 AND 25

SEX	SUBJECT	FIBRINOGEN	ALBUMIN	GLOBULIN	TOTAL PROTEIN
		grams per cent	*grams per cent*	*grams per cent*	*grams per cent*
Males	A	0.17	4.42	2.60	7.19
	B	0.22	3.98	2.50	6.70
	C	0.17	4.02	2.67	6.86
	D	0.21	4.25	3.14	7.60
	E	0.22	4.29	2.51	7.02
Females	A	0.26	4.33	3.41	8.00
	B	0.25	4.09	2.67	7.01
	C	0.23	4.29	2.72	7.24

BLOOD PLASMA PROTEIN VALUES FOUND IN THE NORMAL AND IN CERTAIN PATHOLOGICAL CONDITIONS

Determinations were run on five normal males and three normal females between the ages of twenty and twenty-five. The results are presented in table 6.

In table 7, blood plasma protein values as found in the course of certain pathological conditions in six patients are presented.

TABLE 7

BLOOD PLASMA PROTEINS IN THE COURSE OF CERTAIN PATHOLOGICAL CONDITIONS. SIX CASES CITED

DATE	FIBRINOGEN	ALBUMIN	GLOBULIN	TOTAL PROTEIN	DIAGNOSIS
	grams per cent	*grams per cent*	*grams per cent*	*grams per cent*	
11- 9-36	0.21	1.90	1.54	3.65	Nephritis
11-18-36	0.25	2.38	2.13	4.75	
12- 8-36		2.35	1.77	4.22	
12-13-36	0.27	2.57	2.65	5.49	
12-28-36	0.54	2.37	2.86	5.77	
1-20-37	0.37	2.87	2.92	6.16	
9-11-36		2.26	1.70	4.03	Acute nephritis (autopsy performed)
9-24-36	0.30	2.26	2.10	4.66	
11-16-36	0.31	2.63	2.42	5.36	
11-29-36	0.32	2.76	2.62	5.70	
12-13-36	0.30	2.63	2.14	5.07	
10-19-36	0.44	2.07	2.39	4.90	Acute nephritis
11-28-36	0.30	3.40	2.10	5.80	
12-13-36	0.27	3.16	2.49	5.92	
12-28-36	0.29	3.15	2.33	5.77	
	0.63	0.67	3.00	4.30	Nephritis and severe edema
12-17-36	0.44	2.03	2.86	6.33	Diabetes, liver disease
1- 7-37	0.51	2.02	5.58	7.60	
1-14-37		2.40	4.88	7.28	
1-25-37	0.72	3.00	5.40	9.12	
2- 1-37	0.72	2.51	4.87	8.10	
2-18-37	0.64	2.70	4.80	8.14	
9-10-36	0.38	3.23	6.39	10.00	Multiple myeloma (autopsy performed)
9-26-36	0.45	3.05	8.09	11.59	

CEREBROSPINAL FLUID PROTEINS

Since the method presented has been employed, quantitative spinal fluid protein determinations have been done routinely on all patients admitted to the Department of Neurology and a few on

those admitted to the other departments of the hospital. The values for total protein found in the first 500 determinations are presented in graphic form (fig. 1).

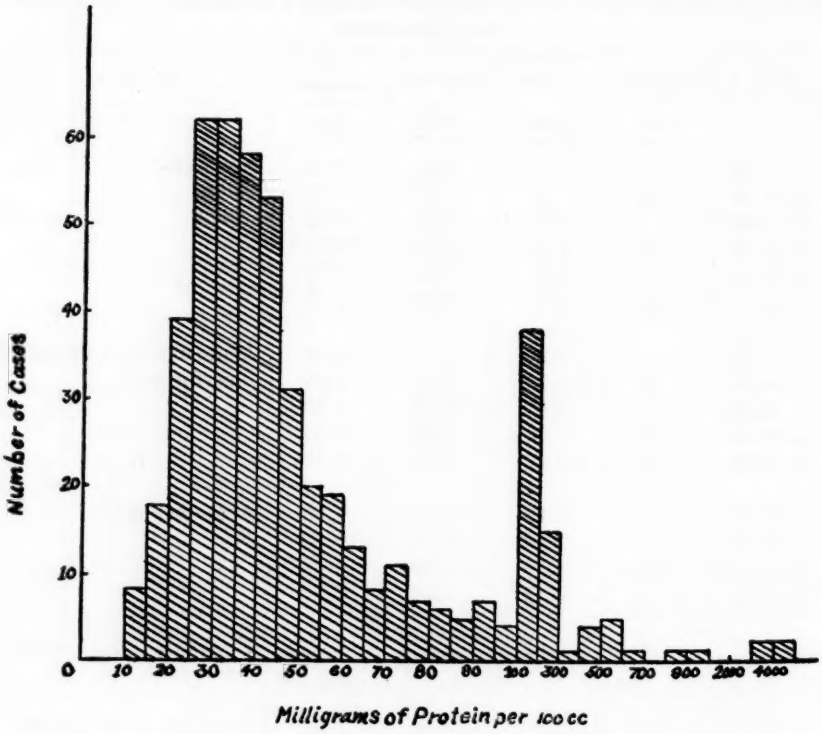


FIG. 1. DISTRIBUTION OF TOTAL SPINAL FLUID PROTEIN IN A SERIES OF 500 CASES

CONCLUSION

Certain modifications in the colorimetric procedure for the determination of blood plasma and spinal fluid proteins are presented with values found in normal and pathological conditions in both instances. The technique is simpler and the results even more constant than in recent methods suggested for these determinations.

REFERENCES

(1) Andersch, Marie, and Gibson, R. B.: The Colorimetric Determination of Plasma Proteins. J. Lab. and Clin. Med., **18:** 816–820. 1933.

(2) Cameron, A. T., Guthrie, J. S., White, F. T.: Estimation of Proteins of Blood Plasma. Canadian Med. Assoc. Journal, **35:** 32–37. 1935.

(3) Cullen, G. E., and Van Slyke, D. D.: Determination of the Fibrin, Globulin, and Albumin Nitrogen of Blood Plasma. J. Biol. Chem., **41:** 587–597. 1920.

(4) Folin, O., and Ciocalteu, V.: On Tyrosine and Tryptophane Determinations in Proteins. Ibid., **73:** 627–650. 1929.

(5) Greenberg, D. M.: The Colorimetric Determination of Serum Proteins. Ibid., **82:** 545–550. 1929.

(6) Greenberg, D. M., and Mirolubova, T. N.: Modifications in the Colorimetric Determination of the Plasma Proteins by the Folin Phenol Reagent. Jour. Lab. and Clin. Med., **21:** 431–434. 1936.

(7) Howe, P. E.: The Use of Sodium Sulfate as the Globulin Precipitant in the Determination of Proteins in Blood. J. Biol. Chem., **49:** 93–107. 1921.

(8) Howe, P. E.: The Determination of Fibrinogen by Precipitation with Sodium Sulfate Compared with the Precipitation of Fibrin by the Addition of Calcium Chloride. Ibid., **57:** 235–240. 1923.

(9) Minot, A. S., and Keller, M.: A Modification of the Greenberg Technic for the Colorimetric Determination of Serum Proteins. Jour. Lab. and Clin. Med., **21:** 743–751. 1936.

(10) Wu, H.: A New Colorimetric Method for the Determination of Plasma Proteins. J. Biol. Chem., **51:** 33–39. 1922.

A NEW CONTRAST MOUNTING MEDIUM IN DIFFERENT COLORS

GUSTAV WILENS

Torrington, Conn.

In the process of bacteriological staining one frequently resorts to the use of counter-stains for producing contrasting effect, the most commonly used are methylene blue, Bismark brown, and safranin. In the Pappenheim panoptic blood stain the mounting medium, euperal, permits the observation of finer detail in the examination of white blood cells. Euperal is a thick, blue green, transparent medium.

In attempting to develop a mounting medium adapted to pigmentation with various dyes and still keeping the transparency and refraction of the medium as close to those of glass as possible the author found the ordinary Dupont cement satisfactory.

The cement is thinned with an equal part of acetone and a minute amount of the dye is added and the mixture shaken vigorously. Quicker, and perhaps better, results are obtained by adding the dye to acetone and using this in thinning the cement.

The finished material is put into collapsible, metal tubes and sealed with a screw cap. This prevents dehydration and possible deterioration of the dye from exposure to light.

The entire list of the biological dyes have been used in this manner and relatively few give an opaque medium. With neutral red a clear red cement is produced which when used to mount stained blood films makes the blue color of stippling, reticulocytes, and basophilia more distinct.

In addition to the above the colored cement may be employed in making color filters such as are used in photomicrography. It is easily applied to glass but rapid manipulation is necessary since it hardens quickly.

PUBLISHED PROCEDURES RECOMMENDED FOR TRIAL

AN ADAPTATION OF VAN SLYKE'S SIMPLIFIED DETERMINATION OF BLOOD UREA CLEARANCE

C. A. PONS

Long Branch, N. J.

Principle: The urine is diluted to such an extent that if the clearance, either standard or maximum, is the average for a normal subject, the urea concentrations in the urine or diluted blood will be equal. The urea in both blood and urine is converted into ammonia with urease, proteins are removed, and the ammonia contents of the two filtrates are compared colorimetrically. A single reading gives directly the average normal renal function in terms of the clearance.

Reagents: 1. N/12 sulphuric acid.

2. 10 per cent sodium tungstate.

3. Urease paper prepared as follows: 15 grams of jack bean meal is shaken with 5 grams of permutit in 100 cc. of 15 per cent alcohol for fifteen minutes using a mechanical shaker or one-half an hour by hand. Filter in ice box over night. Draw pieces of heavy, ammonia-free paper through it and hang to dry. Cut in pieces 1 by 1 by ½ inches.

4. Phosphate buffer solution: dissolve 14 grams of sodium pyrophosphate in N/2 phosphoric acid to make 100 cc. The N/2 phosphoric acid is made by diluting 85 per cent phosphoric acid to one liter and titrating with N/10 alkali using phenolphthalein to a faint pink. Correct as needed.

5. Nessler's reagent.

Procedure: Preferably a few minutes before the test the patient is given a glass of water and asked to empty the bladder. After one hour the urine is collected and a sample of blood is obtained. One hour after that a second specimen of urine is taken. The two specimens of urine are carefully measured and mixed. Timing of the collections of the urine must be exact and if necessary the patient must be catheterized in order to be sure that there is no residual urine in the bladder.

1. Place 10 cc. of urine in a flask and add.

2. 2.0 grams of permutit, shaking at intervals for ten minutes then filter. (if the urine shows more than a heavy trace of albumin add 2.5 grams of permutit instead of the usual 2.0 grams.

3. Place 2.0 cc. of the filtrate in a flask and add

4. 3.2 cc. of N/12 sulphuric acid and

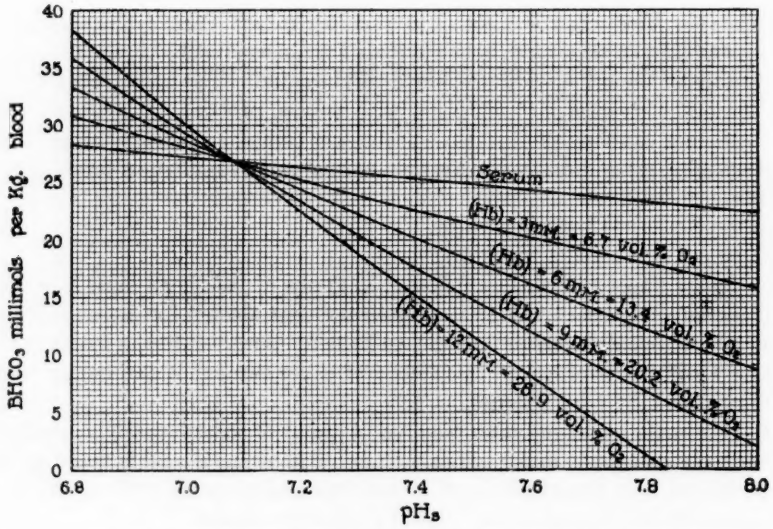
5. Add 14.4 cc. of distilled water and allow to stand for a few minutes

6. Add 0.4 cc. of 10 per cent sodium tungstate and allow to stand for a few minutes.

7. Filter.

Having estimated the number of cc. per hour, the number of times that 1.0 cc. of the urine filtrate is to be diluted can be readily obtained by consulting the dilution chart.

Diluted urine filtrate 5 cc.	Folin-Wu blood filtrate 5 cc.
Urease paper 2 strips	Urease paper 2 strips
Phosphate buffer 3 drops	Phosphate buffer 3 drops



URINE DILURATION CHART (AFTER PETERS AND VAN SLYKE)

Ex. If the urine volume is 80 cc. per hour, the urine according to the chart should be diluted 46.8 times. If, however, the subject is a child of 1.40 meters height, the 80 cc. observed hours volume is multiplied by the factor 1.49. The corrected volume 119 cc. is then interpolated on the curve of the chart. In this case the dilution should be only 37.5 times.

Incubate at 45 to 50 degrees Centigrade for 15 minutes. Transfer quantitatively to test tubes or cylinders, dilute to 22.5 cc. mark

Nesslerize to 25.0 cc. mark.

Compare in the colorimeter.

Calculation: Percentage of average normal clearance equals

$$\frac{100 \times \text{B (reading of blood filtrate)}}{\text{U (reading of urine filtrate)}}$$

If the clearance is less than half the normal average, the blood filtrate when Nesslerized will be more than twice the color of the urine filtrate, then a fresh portion of the blood filtrate is diluted two, three, five, or ten times in order to

bring the B-U ratio to about the same color. In such instances the calculation becomes: Percentage of average normal clearance equals

$$\frac{100 \times B}{\text{Dilution of blood U.}}$$

REFERENCES

(1) VAN SLYKE, DONALD D., AND PETERS, JOHN P.: Quantitative Clinical Chemistry, Williams & Wilkins Co., Baltimore, 1931.

(2) VAN SLYKE, DONALD D., AND COPE, C. L.: Simplified Colorimetric Determination of Blood Urea Clearance. Proc. Soc. Exper. Biol. & Med., **29:** 1169. 1932.

(3) KARR, WALTER J.: Determination of Blood Urea Clearance by Direct Nesslerization of Folin-Wu Filtrate. J. Lab. & Clin. Med., **9:** 329. 1924.

A SIMPLE METHOD FOR SILVER IMPREGNATION OF RETICULIN

GORDON, H., AND H. H. SWEETS, JR. Amer. J. Path., **12:** 545. 1936

1. Fix in formalin or Bouin's fluid.
2. Paraffin, celloidin, or frozen sections.
3. Fix on slide with celloidin or gelatin.
4. Oxidize in acidified potassium permanganate solution: 1–5 minutes.
   (0.5 per cent aqueous potassium permanganate..... 47.5 cc.
   3.0 per cent sulphuric acid..... 2.5 cc.)
5. Wash briefly in water.
6. Bleach in 1 per cent oxalic acid.
7. Wash in rapid succession in tap water and 2–3 changes of distilled water
8. Mordant in 2.5 per cent iron alum: 15 minutes to 2 hours.
9. Wash thoroughly in 2–3 changes of distilled water.
10. Silver hydroxide of Kubie & Davidson until transparent. This may occur instantly but usually a longer time is necessary.

    (*Silver Hydroxide Solution:* To 5 cc. of a 10.2 per cent aqueous solution of silver nitrate add strong ammonia drop by drop until precipitate is just dissolved. Add 3.1 per cent sodium hydroxide. Dissolve the resultant precipitate with 1 or 2 drops of strong ammonia. Dilute to 50 cc. with distilled water.)
11. Wash in distilled water. If impregnation was prolonged wash for a longer period.
12. Reduce in 10 per cent aqueous formalin moving sections to and fro while in the solution. Reduction takes place almost immediately.
13. Wash in tap water, tone if desired in 0.2 per cent aqueous gold chloride until purplish, fix in 5 per cent sodium thiosulphate and wash in 2 or more changes of tap water.
14. Dehydrate, clear, and mount.

STAIN FOR MYELIN SHEATHS IN TISSUES EMBEDDED IN PARAFFIN

GEORGE S. MAHON, Arch. Neurol. & Psychiat., **38:** 103. 1937

Reagents: 1. Four per cent iron alum (iron and ammonium sulfate).

2. Saturated solution of lithium carbonate in distilled water.

3. Ten per cent solution of hemotoxylin in absolute alcohol.

Technic: 1. Fix thoroughly in 10 per cent formalin, either neutralized or unneutralized is satisfactory.

2. Embed in paraffin. Cut 6 to 18 microns, mount on slides with albumin glycerin, deparaffinize and run down to water.

3. Mordant 15 to 30 minutes in 4 per cent iron alum at room temperature.

4. Rinse thoroughly (a half minute) in distilled water

5. Stain 30 to 60 minutes in lithium carbonate hemotoxylin prepared as follows:

Reagents:

A. A ten per cent hemotoxylin in absolute alcohol

B. A saturated aqueous solution of lithium carbonate, 7 cc.
Distilled water q. s., 100 cc.

Stain:

Solution A. 1 part.

Solution B. 9 parts.

The staining solution must be mixed fresh each time.

6. Wash in distilled or tap water to remove excess stain

7. Leave in saturated aqueous solution of lithium carbonate 15 to 30 minutes to blue the myelin sheaths

8. Wash thoroughly, dehydrate in 95 per cent and absolute alcohol, clear in xylene and mount in neutral xylene balsam.

Note: Pyroxylin sections stain as well as paraffin ones except for a slight bluish tone in the gray matter. The method is not suitable for frozen sections and is not suitable for study of cortical myelo-architectonics.

A SIMPLE METHOD FOR OBTAINING ANAEROBIOSIS

J. E. WEISS AND E. H. SPAULDING, J. Lab. & Clin. Med., **22:** 726. 1937

By modifying the methods of McIntosh and Fildes, Eggerth and Gagnon, and Boëz the following simplified procedure was found to be efficient and practical.

A Hempel type dessicator having a capacity of ten Petri plates or forty $\frac{5}{8}$ inch tubes and fitted with a glass stopcock is used. In it is placed a small flat porcelain dish containing 0.4 grams of palladinized asbestos. The ground edges are coated with vaseline, or better with Cello-Seal (Fisher), and the jar is connected by means of a three-way stopcock with a system containing a Cenco-Hyvac pump, a manometer and a hydrogen tank. It is evacuated to the de-

sired negative pressure, the pump is disconnected by means of the stopcock and hydrogen is passed in until atmospheric pressure is restored. A jar containing Petri plates can be evacuated safely to a negative pressure of 720 mm. of mercury while with liquid media 740 mm. is usually reached before bubbling begins.

If carbon dioxide is desired it may be introduced before the addition of the hydrogen by attaching the system to a tank of the gas.

The catalyst gradually loses its efficiency through absorption of moisture but its full activity may be restored by heating for a few minutes over a Bunsen flame.

INDIVIDUAL CULTURE DISH WITH INCREASED CARBON DIOXIDE TENSION

A. L. Joyner and C. P. Jones, J. Lab. & Clin. Med., **22:** 1184. 1937

Combining the Spray anaerobic culture dish and the Thompson method for supplying known amounts of carbon dioxide furnishes an ideal system for growing those organisms which demand a lowered oxygen tension.

Place 1 cc. of a molar solution of sodium bicarbonate (84 grams to one liter of distilled water) in one compartment of the dish and in the other 1 cc. of a solution of sulphuric acid (1 cc. of concentrated acid to 29 cc. of distilled water).

The innoculated Petri plate is sealed on the Spray jar by means of melted paraffin and after the paraffin has hardened the jar is tilted just enough to mix the two fluids thoroughly. The above amounts of the two reagents will produce, according to Thompson, 22.4 cc. of carbon dioxide. Since the Spray dish has a capacity of something over 200 cc. there is, at the completion of the reaction, a carbon dioxide concentration of 10 per cent.

The combination provides a quick, easy method for preparing individual cultures. It is especially valuable in the isolation of members of the genus *Neisseria*.

A RAPID CULTURAL METHOD FOR THE DIAGNOSIS OF TINIA INFECTIONS

F. L. Blumenthal and J. S. Snow, J. A. M. A., **107:** 1367. 1936

A deep hanging drop slide is washed thoroughly, allowed to dry and flamed. Cover glasses in alcohol-ether mixture are dried by passing through the flame.

By means of a sterile loop a small drop of the following culture medium is placed in the center of a cover glass:

Culture medium:

Crude maltose of Chanut	4 grams
Peptone of Chassaing	1 gram
Distilled water to make	100 cc.

The specimen of skin scrapings or hairs is added to the drop of culture medium and the cover glass is inverted, placed on the slide and sealed in place with melted paraffin applied to the edges by means of a heated knife.

The preparation is incubated at room temperature and examined twice a day with the microscope.

In 48 cases diagnosed clinically as tinia infections 16 were positive by microscopic examination of specimens treated with 20 per cent potassium hydroxide; 31 were positive by culture on Sabauraud's agar with the average time of appearance of the growth 6.1 days while with the hang drop preparation there were 35 positive cases with the average time of appearance of only 1.8 days.

ANNOTATIONS, MINOR CONTRIBUTIONS, QUERIES

MAYER'S ALBUMEN. The directions generally given for the preparation of this mixture call for filtering, but this is a difficult and time consuming procedure. A simple expedient makes it easy and rapid. Place the ingredients (Albumen 50 cc., glycerin 50 cc. and sodium salicylate 1 gram) in a 250 cc. cylinder, shake vigorously and allow to stand for a few minutes. The foam produced by the shaking will come to the top and bring with it the fragments of membrane it is desired to remove. The clear mixture below may then be removed by means of a pipette. (Slightly modified from McClung, Microscopical Technique, 2nd. ed., 1937.)

LUGOL'S SOLUTION. To prepare, add only sufficient water to the potassium iodide to dissolve it (1 cc. to 1 gram). The iodine will go into solution almost immediately. Add the remaining water and the solution is ready for use.

This note is prompted by a recent experience with a well trained technician who had always made the solution the long way by pouring the total quantity of water into a flask containing the iodine and potassium iodide. The point, of course, is that iodine is readily soluble in a saturated aqueous solution of potassium iodide, dissolves slowly in a weak solution of the salt, and practically not at all (1:5000) in water alone.

STAINING NASAL SMEARS FOR EOSINOPHILE COUNTS. Stain with Wright's stain as with blood, wash and decolorize 2 to 3 minutes with 95 per cent alcohol, wash and stain with Loeffler's alkaline methylene blue. Eosinophiles stand out as bright pink while the neutrophiles are blue. (Irene Weiber, J. Lab. & Tech., **22:** 1286. 1937).

HANDY SLIDE AND COVERSLIP RACK. Cartridges of staples manufactured by the E. H. Hotchkiss Co. for their No. 1 Automatic Paper Fastener form a cheap and handy rack for slides and coverslips. They may be simply laid on their backs on the table or nailed to small boards. (F. W. Cooper, St. Tech., **11:** 166. 1936.)

A METHOD FOR LOOSENING STUCK SYRINGES. The simple method following was described by J. B. Hopkins, M.D. in the Amer. Med. Assn. Journal correspondence (February 6, 1937).

The smallest syringe at hand (tuberculin or 2 cc.) is half filled with water and a one-half inch hypodermic needle attached. The needle is then thrust through a thickness of rubber, such as a rubber band, inserted into the tip of the stuck

syringe, and pressure made on the plunger. The force is transmitted to the stuck plunger. The smaller the area of the free plunger, the greater the hydraulic force transmitted to the stuck plunger.

Some care must be used with badly stuck syringes.

A NEW MATERIAL TO PREVENT CULTURE MATERIAL FROM DRYING. A new product ("Parafilm") manufactured by the Menasha Products Co., Menasha, Wis.) has been found of value in the preservation of culture media (in slants, flasks, or plates). It is also useful in preventing stock cultures from drying out. After the cotton plug has been pushed down, a square of the film is pressed down on the mouth of the culture tube. The seal is readily stripped off for examination of the culture and the culture plug remains perfectly manageable.